

# A photostable fluorescent probe for targeted imaging of tumour cells possessing integrin $\alpha_v\beta_3$ \*\*†

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**A one-step  $S_NAr^H$  reaction has been used for the synthesis of photostable probe **2**, which has good water solubility and low cytotoxicity; this probe can be used for targeted imaging of tumour cells by virtue of specific binding between integrin  $\alpha_v\beta_3$  and an arginine–glycine–aspartic acid tripeptide sequence.**

Fluorescence imaging is emerging as a powerful tool in bioscience and medicine because it provides a facile method for studying diseases at the cellular and molecular level.<sup>1</sup> Recently, the targeted imaging of specific molecular structures using fluorescent probes has attracted much interest, such as targeted imaging of the cell adhesion molecule integrin  $\alpha_v\beta_3$ .<sup>2–5</sup> Integrin  $\alpha_v\beta_3$  is a viable target for non-invasive imaging of invasive tumours, it is significantly up-regulated in activated endothelial cells and fast-growing solid tumour cells compared to its minimum expression in quiescent blood vessels and most normal tissues. Integrin  $\alpha_v\beta_3$  binds extracellular matrix molecules such as vitronectin, which contain the amino acid sequence Arg–Gly–Asp (RGD).<sup>3</sup> For these reasons, many molecular fluorescent probes containing the RGD motif, including organic probes such as the Cy dyes,<sup>4</sup> and semiconductor quantum dots (QDs),<sup>5</sup> have been developed for targeted imaging of integrin  $\alpha_v\beta_3$  expression. However, for application in biological fields, an ideal probe should display water-solubility, low cytotoxicity, cell-permeability and photostability.<sup>6</sup> Unfortunately, conventional organic dyes are still prone to photobleaching, which limits their extensive use and efficient application,<sup>7</sup> while QDs are controversial due to their inherent toxicity and chemical instability.<sup>8</sup> Therefore, it is necessary to develop fluorescent probes with low cytotoxicity and high photostability.

As a novel electron-deficient heterocycle, 8-oxo-8*H*-acnaphtho[1,2-*b*]pyrrole-9-carbonitrile **1** (Scheme 1) can react with N, O, or S nucleophiles by nucleophilic aromatic substitution ( $S_NAr^H$ ).<sup>9</sup> By virtue of its reaction with the –SH of cysteine/homocysteine, **1** has recently been used as a fluorescent sensor for Cys/Hcy, showing a 75-fold fluorescence enhancement.<sup>9b</sup> In the work described herein, the non-fluorescent compound **1** has been easily converted into the intensely

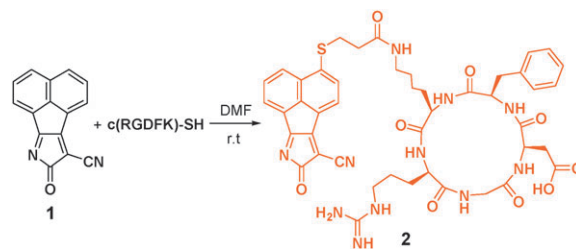
fluorescent probe **2** by means of an  $S_NAr^H$  reaction with the thiolated RGD peptide cyclo(Arg–Gly–Asp–Phe–Lys(mpa)) (c(RGDFK)-SH). Importantly, probe **2** exhibits good water solubility and low cytotoxicity, and furthermore can be used in the targeted imaging of integrin  $\alpha_v\beta_3$  expression in tumour cells.

Probe **2** was easily synthesized by stirring **1** and c(RGDFK)-SH in DMF for 2 h at room temperature, and was purified by reversed-phase preparative HPLC. The purity of **2** was 95.5% according to analytical reversed-phase HPLC analysis (Fig. S1†). The HPLC retention time of **2** was found to be 6.028 min ( $C_{45}H_{49}N_{11}O_9S$ , calculated 920.34; found 920.3 [ $M + H$ ]<sup>+</sup>).

As shown in Fig. 1, probe **2** exhibits a broad UV-vis band with a maximum at a wavelength of 512 nm, which may be assigned to intramolecular charge-transfer between the SH (donor) and carbonitrile (acceptor). It should be noted that an obvious red-shift of 74 nm was observed for the maximum absorption wavelength of **2** in comparison with that of **1**, corresponding to an apparent colour change from yellow-green to red (Fig. 1). Probe **2** emitted a distinctive orange-red fluorescence ( $\lambda_{max}^{em} = 570$  nm) under excitation with visible light at 515 nm. Importantly, compared with non-fluorescent compound **1** ( $\phi < 0.1\%$ ), the quantum efficiency of **2** in DMSO–phosphate buffer solution (PBS) (pH 7.3; 1 : 119, v/v) was 0.11 with reference to rhodamine B as a standard.<sup>10</sup>

To investigate the cytotoxicity of **2**, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with the human hepatocellular carcinoma cell line SMMC-7721 was used to determine the effect of **2** on cell proliferation after 24 h. No significant differences in the proliferation of the cells were observed in the absence or presence of 5–100  $\mu M$  **2** (Fig. 2). The cellular viabilities were estimated to be greater than 85% after 24 h. These data show that **2** ( $\leq 100 \mu M$ ) can be considered to have low cytotoxicity. The good water solubility and low cytotoxicity implied that **2** could serve as a potential fluorescence probe for targeted imaging.

To demonstrate that **2** can indeed act as a specific ligand for the  $\alpha_v\beta_3$  integrin receptor, the binding and subcellular



Scheme 1 Synthesis of **2**.

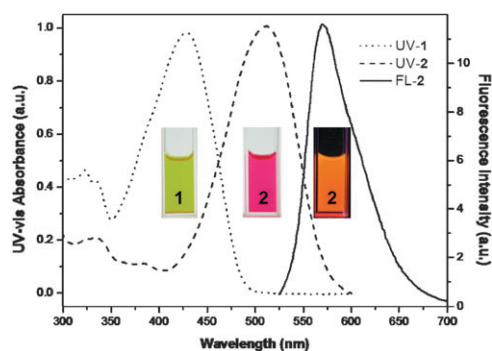
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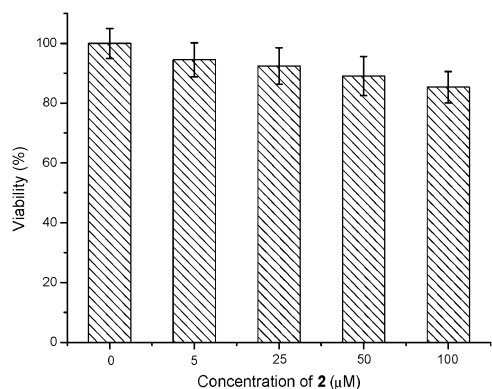
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<sup>b</sup> The Obstetrics and Gynecology Hospital, Fudan University, Shanghai, 200433, P. R. China. E-mail: xucj@hotmail.com

† Electronic supplementary information (ESI) available: Experimental procedures and characterization of **2**. Movie showing the photostability of **2**. See DOI: 10.1039/b820576k

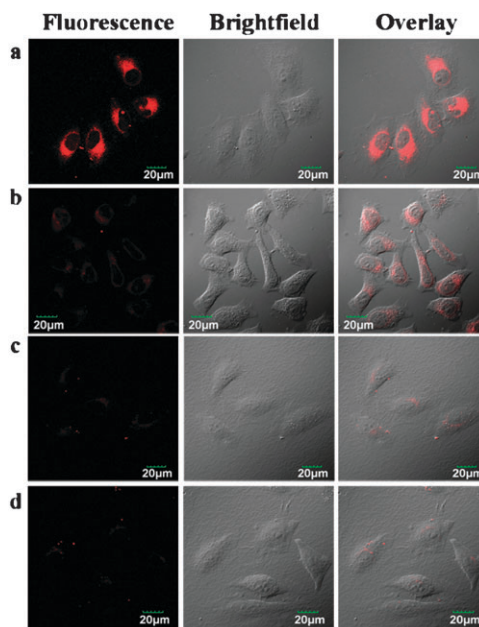


**Fig. 1** UV-vis absorption spectra of **1** and **2** in DMSO (10  $\mu\text{M}$ ) and the room temperature fluorescence spectrum ( $\lambda_{\text{ex}} = 515 \text{ nm}$ ) of 5  $\mu\text{M}$  **2** in DMSO–PBS (1 : 119, v/v). Photographs (inset) show the corresponding colour difference between **1** and **2**, the fluorescence photograph of **2** was obtained under 365 nm UV excitation.



**Fig. 2** Cell viability values (%) estimated by MTT proliferation test *versus* incubation concentrations of **2**. Cells were incubated with 5–100  $\mu\text{M}$  **2** at 37  $^{\circ}\text{C}$  for 24 h.

localization of **2** were examined using an OLYMPUS FV1000 laser scanning microscope. We chose two cell lines, human cervical carcinoma HeLa cells,<sup>11</sup> which are known to possess integrin  $\alpha_v\beta_3$ , and human hepatocellular carcinoma SMMC-7721 cells as a control. As shown in Fig. 3, a strong fluorescence signal was observed in the HeLa cells (Fig. 3a), whereas the SMMC-7721 cells showed much less fluorescence (Fig. 3b). Moreover, the fluorescence intensity of **2** obtained from the live HeLa cells was higher than that obtained from the SMMC-7721 cells (Fig. S2<sup>†</sup>). These results imply that the HeLa cells express a higher amount of integrin  $\alpha_v\beta_3$  than the SMMC-7721 cells. It is known that integrin  $\alpha_v\beta_3$  receptors can be rapidly transported through the early endosomes, arriving at the perinuclear compartments approximately 30 min after endocytosis.<sup>12</sup> This phenomenon is consistent with our observation of the accumulation of **2** in the perinuclear region after 30 min of incubation at 37  $^{\circ}\text{C}$  (Fig. 3a). Such an endocytosis mechanism can be blocked at 4  $^{\circ}\text{C}$ . In a separate experiment, the HeLa cells were therefore loaded with **2** at 4  $^{\circ}\text{C}$  for 30 min and, as expected, only weak fluorescence was observed (Fig. 3c). This result supports the view that **2** is likely to enter the cells by this endocytotic uptake mechanism. The integrin receptor specificity of **2** could be further confirmed by a competition assay. The HeLa cells were pre-incubated with a tenfold excess of unlabelled c(RGDFK) peptide at 37  $^{\circ}\text{C}$  for



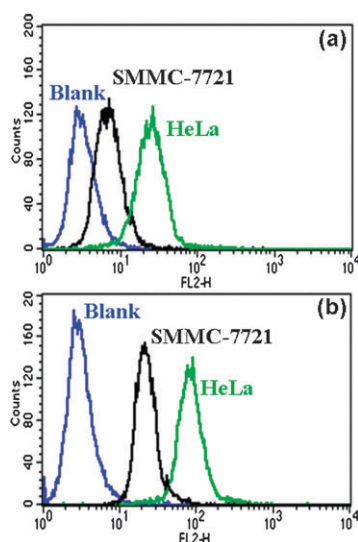
**Fig. 3** Confocal fluorescence images of 20  $\mu\text{M}$  **2** incubated with HeLa cells (a) or SMMC-7721 cells (b) for 30 min at 37  $^{\circ}\text{C}$  in DMSO/PBS (1 : 119, v/v). Incubation with HeLa cells at 4  $^{\circ}\text{C}$  (c) or in the presence of unlabeled c(RGDFK) peptide (d) were also shown.

30 min and then incubated with **2** at 37  $^{\circ}\text{C}$  for 30 min. As a result, almost no fluorescence signal was observed (Fig. 3d).

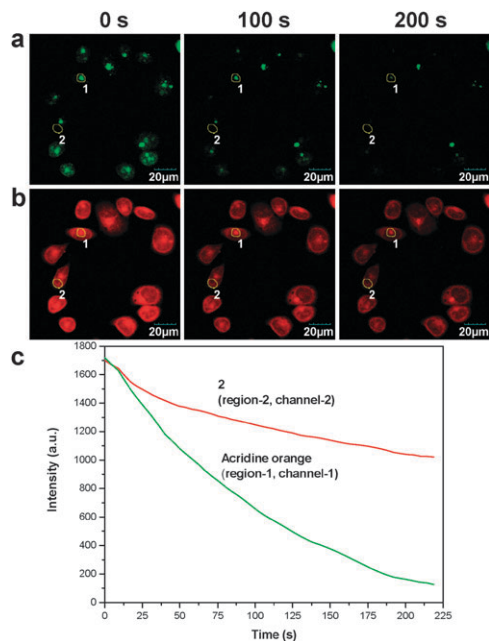
Additionally, the cellular uptake characteristics of **2** were investigated by means of flow cytometry. Upon excitation at 488 nm, HeLa cells incubated with **2** at 37  $^{\circ}\text{C}$  for 30 min displayed high emission intensities, while the emission intensities of SMMC-7721 cells treated with **2** were lower (Fig. 4a). Increasing the incubation time to 2 h, the HeLa cells still showed a higher uptake of **2** than the SMMC-7721 cells (Fig. 4b), confirming that the HeLa cells express a higher amount of integrin  $\alpha_v\beta_3$  than the SMMC-7721 cells. Thus, the results of confocal microscopy and flow cytometry studies demonstrated that **2** can target tumour cells possessing integrin  $\alpha_v\beta_3$ .

As is well known, the photobleaching characteristics of fluorescent materials are critical to their application as bio-probes. In the present study, the photostability of **2** was investigated by comparison with that of acridine orange<sup>6</sup> (an organic dye) under the same excitation conditions. To distinguish their staining regions, **2** and acridine orange were chosen to stain the cytoplasm and nuclei, respectively, of live SMMC-7721 cells. After continuous excitation at 488 nm with a high focal power (1.1 mW) for 200 s, the fluorescence intensity of acridine orange (520  $\pm$  20 nm, region-1, channel-1) decreased to 6% of its initial value, as shown in Fig. 5 (Fig. S3 and movie<sup>†</sup>). In contrast, the fluorescence intensity of **2** (580  $\pm$  20 nm, region-2, channel-2) still remained at 60% of the original value during the same period of excitation. These data established that **2** was more photostable than acridine orange. Furthermore, the chemical stability of **2** was confirmed by the fact that the stable fluorescence intensity in DMSO–PBS (1 : 119, v/v) was kept for a few days (Fig. S4<sup>†</sup>).

In conclusion, we have developed a photostable fluorescent probe **2** that was synthesized by the one-step  $\text{S}_{\text{N}}\text{Ar}^{\text{H}}$  reaction



**Fig. 4** Flow cytometric histogram profiles of cellular uptake of **2** in HeLa cells and SMMC-7721 cells. The *x* axis indicates the log of the fluorescence intensity and the *y* axis indicates the number of cells. Cells were incubated with 20  $\mu\text{M}$  **2** in DMSO–PBS (1 : 119, v/v) at 37  $^{\circ}\text{C}$  for 30 min (a) and 2 h (b). Blue line indicates the emission intensities of SMMC-7721 cells in the absence of **2**.



**Fig. 5** Comparison of **2** and acridine orange for resistance to photobleaching. Confocal fluorescence images of SMMC-7721 cells stained with **2** (a) and acridine orange (b) under continuous excitation at 488 nm with different laser scan times (0, 100, 200 s). (c) Fluorescence decay curves of **2** and acridine orange during the same period. The signals of acridine orange and **2** were collected from the region-1 of channel-1 (520  $\pm$  20 nm) and region-2 of channel-2 (580  $\pm$  20 nm), respectively. Cells were incubated at 37  $^{\circ}\text{C}$  for 1 h.

of **1** and thiolated RGD peptide c(RGDFK)-SH. Probe **2** has good water solubility and low cytotoxicity. Moreover, through specific binding of integrin  $\alpha_v\beta_3$  to the RGD tripeptide sequence, **2** can be used for targeted imaging of tumour cells possessing integrin  $\alpha_v\beta_3$ . The findings open up new perspectives

for integrin-targeted fluorescence imaging and suggest that **2** has great potential in imaging-guided cancer diagnosis.

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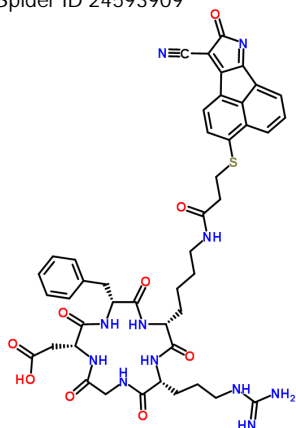
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## Primary compounds

SNRQMMAXXKPLKK-SEVDZJIVSA-N

compound no. 2

ChemSpider ID 24593909

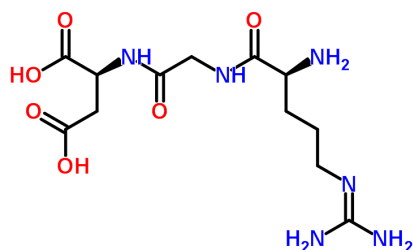


## Secondary compounds

IYMAXBFPHPZYIK-BQBZGAKWSA-N

RGD

ChemSpider ID 94603

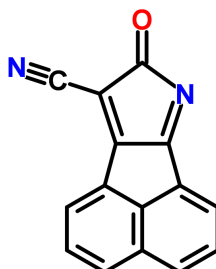


VQYSTSIAUDPRJH-UHFFFAOYSA-N

8-oxo-8H-acenaphtho[1,2-b]pyrrole-9-carbonitrile

compound no. 1

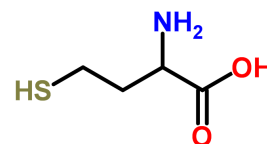
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FFFHZYDWPBMWHY-UHFFFAOYSA-N

homocysteine

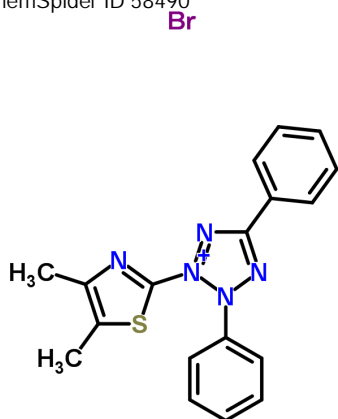
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AZKSAVLVSZKNRD-UHFFFAOYSA-M

MTT

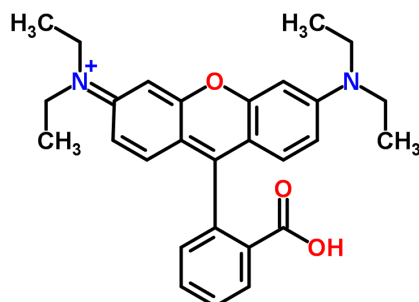
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CVAVMIODJQHEEH-UHFFFAOYSA-O

rhodamine B

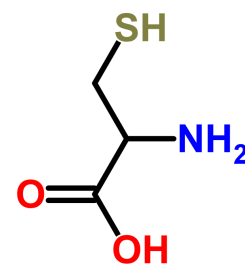
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XUJNEKJLAYXESH-UHFFFAOYSA-N

cysteine

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## Routine compounds

XLYOFNOQVPJJNP-UHFFFAOYSA-N

water

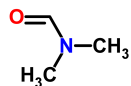
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DMF

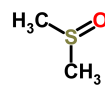
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IAZDPXIOMUYVGZ-UHFFFAOYSA-N

DMSO

ChemSpider ID 659



DPKHZNPWBDQZCN-UHFFFAOYSA-N

acridine orange

ChemSpider ID 56136

